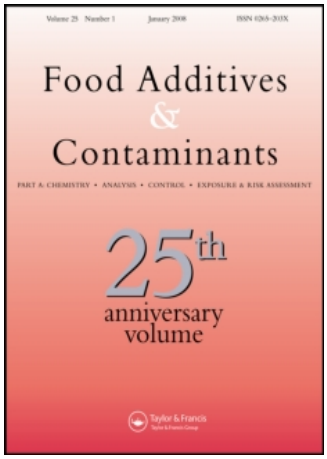

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Development of monoclonal antibodies for the fusarin mycotoxins

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Development of monoclonal antibodies for the fusarin mycotoxins

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Abstract
The fusarins are a group of mycotoxins produced by fungi that commonly infest cereal crops, in particular by the fungus *Fusarium verticillioides*. This group of compounds is characterized by a substituted 2-pyrrolidone ring attached to a 12-carbon polyunsaturated backbone. Several of the fusarins contain an epoxide substitution on the pyrrolidone ring and are highly mutagenic. This paper describes the development of seven monoclonal antibodies and immunoassays for detecting fusarins C and A. Fusarin C was isolated and conjugated to ovalbumin to produce the immunogen. Competitive indirect enzyme-linked immunosorbent assays (CI-ELISAs) were developed based upon the isolated monoclonal antibodies. The concentrations of fusarin C able to inhibit colour development by 50% (IC_{50}) in CI-ELISAs were 1.0, 2.0, 3.6, 23.4, 28.9, 31.4, and 66.7 ng ml^{-1} for clones 1–38, 1–30, 1–5, 1–7, 1–43, 1–25, and 1–21, respectively. Cross-reactivity with fusarin A was 44.8, 51.4, 41.1, 174.0, 62.6, 78.2, and 98.0% for clones 1–38, 1–30, 1–5, 1–7, 1–43, 1–25, and 1–21, respectively. Given the sensitivity of these antibodies for fusarins it is expected that, with further development, they may be useful for detecting fusarins at relevant levels in foods.

Keywords: *Fusarin, mycotoxin, antibody, immunoassay,* Fusarium verticillioides

Introduction

Fusarins are a class of mycotoxins characterized by a 2-pyrrolidone moiety substituted with a pentaene chain (Wiebe and Bjeldanes 1981; Gelderblom et al. 1983, 1984, Gaddamidi et al. 1985; Kim and Bjeldanes 1992) (Figure 1). The fusarins are produced by many *Fusarium* species that commonly infest cereal grains, including *F. verticillioides* (formerly *F. moniliforme*), *F. oxysporum*, *F. sporotrichioides*, *F. poae*, and *F. graminearum* (*F. venenatum*) (Bever et al. 2000; Song et al. 2004; Thrane et al. 2004). The early stages of fusarin biosynthesis are believed to be performed by a type I polyketide synthase (Song et al. 2004; Gaffoor et al. 2005).

Four fusarins (A–D) were originally described by Wiebe and Bjeldanes (1981). Fusarin C was originally isolated based upon bioactivity as a mutagen in the Ames assay and required liver S-9 fraction for activity (Wiebe and Bjeldanes 1981). It has been found as a natural contaminant in visibly mouldy corn and healthy corn kernels from the Transkei region of South Africa (Gelderblom et al. 1984b) and in corn screenings from Pennsylvania, USA (Thiel et al. 1986). Fusarin C may be acted upon by carboxylesterases to yield fusarin PM1 (Figure 1), which is much less mutagenic than fusarin C (Gelderblom et al. 1988; Lu et al. 1988). The early toxicology literature was summarized in an excellent review by Farber and Scott (1989). Fusarin C along with fumonisins B_1 and B_2, which are also produced by *F. verticillioides*, has been classified as a possible human carcinogen by the International Agency for Research on Cancer (group 2B; IARC 1993). Experimental evidence concerning the carcinogenicity of fusarin C is conflicting, as summarized by Bever et al. (2000).

Fusarins A and D lack the epoxide substitution on the pyrrolidone ring and were non-mutagenic in the Ames test. As such, it appears that the ultimate mutagen results from the metabolic activation of the epoxide (Gelderblom et al. 1988). The presence of the pentaene side-chain was also important for

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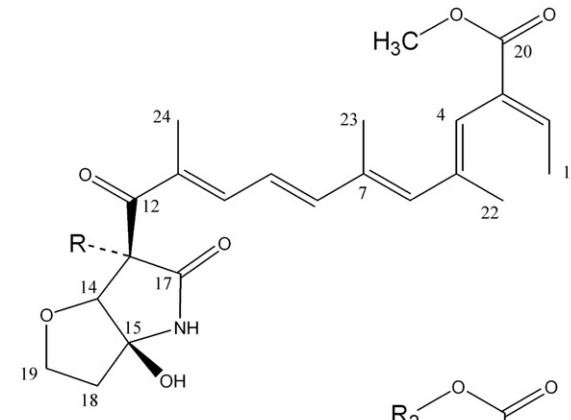
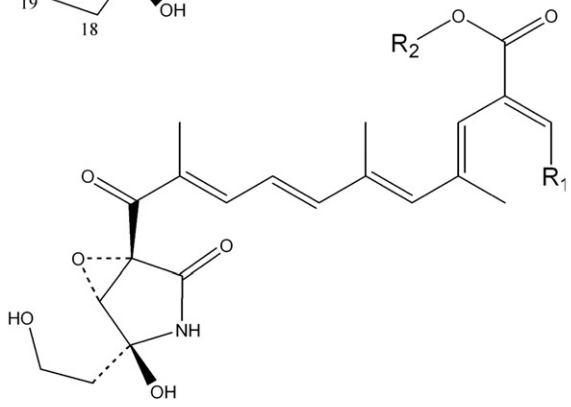
Fusarin	R
A	H
D	OH



Fusarin	R_1	R_2
C	CH_3	CH_3
X	CH_2OH	CH_3
PM1	CH_3	H



Fusarin F

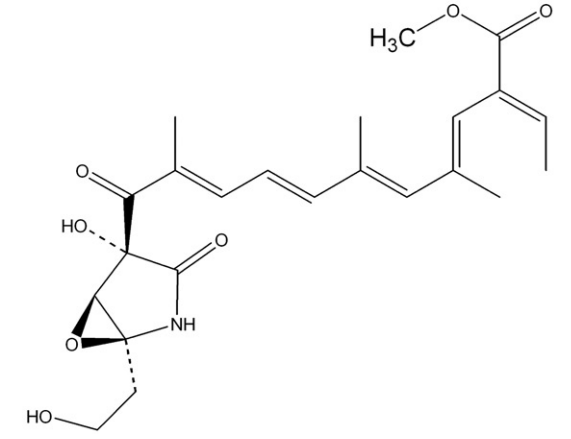


Figure 1. Representative structures of the fusarins.

activity, as fusarin C analogues lacking the chain were only weakly mutagenic (Kim and Bjeldanes 1992).

Fusarin F has the same molecular mass as fusarin C, but differs in the positions of the epoxide and the hydroxyl attached to the pyrrolidone ring (Figure 1) (Savard and Miller 1992). Savard and Miller noted that separation of the fusarin peaks on a reversed-phase column with an aqueous methanol solvent system induced a rearrangement of fusarin F to C. Small amounts of fusarin C were also found to be produced in samples of fusarin F that were left standing. In that report fusarin F was described as less stable than fusarin C, with the equilibrium between the two structures favouring fusarin C.

Fusarin X, the 1-hydroxy derivative of fusarin C, was a potent direct-acting mutagen in the Ames test (Lu and Jeffrey 1993). Fusarin X and fusarin Z, a γ-lactone analogue of fusarin X, could also be formed from fusarin C using an *in vitro* metabolic activation system and were more potent mutagens than fusarin C in the Ames test (Zhu and Jeffrey 1993).

Compounds resembling the fusarins but lacking the 7-methyl group have also been reported. The 7-demethyl analogues of fusarin C and (8Z)-fusarin C were reported to be produced by *Metarhizium*

anisopliae, which may impact the use of this fungus as an agent for biocontrol of insects (Krasnoff et al. 2006). The 7-demethyl analogue of fusarin A, lucilactaene, has been described as a cell cycle inhibitor produced by an unidentified *Fusarium* species (Kakeya et al. 2001).

Fusarin C has been the most studied of the fusarins. However, the nomenclature of fusarin C has differed among various reports. The reports differ in whether the carbon at the end of the pentaene is numbered first (as in Figure 1), the C in the carboxy methyl ester is numbered first (C20 in Figure 1), or the carbonyl within the pyrrolidone ring is numbered first (C17 in Figure 1) (Eilbert et al. 1997). The nomenclature in Figure 1 is the most commonly used and derives from the report of Gelderblom et al. (1984a).

Regardless of the nomenclature there is general agreement in the literature that the double bonds of the pentaene chain can rearrange to form various stereo-isomers. Therefore, while fusarin C is represented in Figure 1 as a single structure, it is possible for several isomers to be present. Jackson et al. (1990) developed a method for purification of fusarin C and noted the formation of three additional isomers during handling and storage. Eilbert et al. (1997) isolated the all *trans*-isomer as well as two additional isomers of fusarin C from *Nectria coccinea*. Furthermore, in the crystal state the 8Z form of fusarin C has an intra-molecular H-bond between the NH16 and the C20 carbonyl (Gelderblom et al. 1984a).

Fusarin C has an absorption maximum near 360 nm and an extinction coefficient ranging from 25 635 to 33 000 depending upon the batch examined and the solvent used (Wiebe and Bjeldanes 1981; Gelderblom et al. 1984a; Gaddamidi et al. 1985; Scott et al. 1986). Fusarin C is unstable to prolonged exposure to ultraviolet (UV) light and to elevated temperatures (Gelderblom et al. 1983, 1984a, b; Scott et al. 1986; Jackson et al. 1990; Bever et al. 2000). The products of exposure of fusarin C in dichloromethane solution to 366 nm light were reported to be the starting material and the 8Z, 6Z, and 10Z stereo-isomers (Gelderblom et al. 1984a). Thus, short exposure to UV light promoted the rearrangement of the stereochemistry of the original configuration. Prolonged exposure to UV light resulted in a loss of the UV absorption band indicating loss of the pentaene chromophore. Losses were reduced by handling the fusarins under 'gold' fluorescent lights (Scott et al. 1986). Prolonged exposure to light resulted in loss of the mutagenic activity and therefore indicated degradation (Gelderblom et al. 1983). Fusarins C and X were also decomposed by glutathione, and by high temperatures, especially at alkaline pH (Scott et al. 1986; Lu and Jeffrey 1993). Glutathione has also been reported to interact with fusarin C resulting in the formation of fusarin A and a compound lacking the 2-pyrrolidone moiety (Gelderblom et al. 1988). The chromophore of fusarin C was also unstable when small amounts (less than 100 μg) were dried under nitrogen (Jackson et al. 1990).

Because of the instability of fusarin C to light, and the possibility of rearrangement to various isomers under both aqueous and non-aqueous conditions, isolation and quantitation of this toxin has been difficult. Normal- and reverse-phase HPLC methods for detection of fusarins in cereal products were reported by Scott et al. (1986). The methods involved extraction with either methylene chloride/acetonitrile or acetonitrile alone, followed by solid-phase extraction (SPE) with silica gel or amino columns. A previous normal-phase HPLC method involved measuring the peaks of the four isomeric forms, summing them, and comparing the sum to the peak area of an internal standard (Jackson et al. 1990). Perhaps because of the instability of the fusarins, antibodies for their detection have not been described. We report the development of seven monoclonal antibodies (Mabs) and enzyme-linked immunoassay (ELISA) systems for the detection of fusarins under aqueous conditions.

Materials and methods

Reagents

Except where noted otherwise, deionized water (Nanopure II, Sybron/Barnstead, Boston, MA, USA) was used in the preparation of all reagents. All solvents were HPLC grade. Chicken egg albumin (ovalbumin, OVA), bovine serum albumin (BSA), polyvinyl alcohol (PVA), 1,1′-carbonyldiimidazole (CDI) and 1[3-(dimethylamino)propyl]-3-ethyl-carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Peroxidase conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other chemicals and solvents were reagent grade or better and purchased from major suppliers.

Fusarin preparations

The fusarin C and fusarin A were produced at the National Center for Agricultural Utilization Research (Peoria, IL, USA). *Fusarium verticillioides* M-3125 was grown in liquid culture for 14 days as described previously (Proctor et al. 1999). Fusarins were extracted with an equal volume of acetonitrile, filtered, and the mixture partitioned twice against

methylene chloride. The organic fraction was concentrated under vacuum to an oil. The oil was solubilized in 50 ml of *n*-hexane and extracted twice with a total of 100 ml of acetonitrile. The extract was concentrated under vacuum and diluted with water to give a solution of approximately 10% (v/v) acetonitrile. This concentrated extract was injected unto a preparative reverse-phase HPLC system having an 8 μ 21.4 × 250 mm Microsorb C18 Dynamax HPLC column (Varian, Walnut Creek, CA, USA) The fusarins were collected as 50 ml acetonitrile/water fractions. The mobile phase (20 ml min^{-1}) was initially 100% water for 2.5 min, after which the acetonitrile was increased to 50% by 60 min. Between 60 and 90 min the acetonitrile concentration was increased to 70%. The fractions were extracted with methylene chloride, concentrated to oils, and stored at −20°C. Fractions were examined by HPLC-MS. The NMRs of the isolated materials were consistent with those of authentic fusarins as reported in the literature (Gelderblom et al. 1984).

Stock solutions of fusarins A and C were prepared by diluting the corresponding oils in acetonitrile. The fusarin concentrations of the stock solutions were calculated from the UV absorbance and the published extinction coefficients of 33 000 at 360 nm in 95% ethanol for fusarin C and 21 300 at 352 nm in methanol for fusarin A (Wiebe and Bjeldanes 1981; Steyn and Vleggaar 1985). The instrument used was a Perkin Elmer (Waltham, MA, USA) model lambda 20 UV/Vis spectrophotometer. Spectra were collected over the range 250–450 nm.

HPLC with photodiode array or mass spectrometric detection

The fusarin C and fusarin A stock preparations were examined by HPLC with either photodiode array (PDA) or mass spectrometric (MS) detection. Reverse-phase HPLC-PDA or HPLC-MS was also used to follow the stability of the fusarin preparations after dilution in buffer. The HPLC-PDA instrumentation consisted of a Beckman Coulter System Gold 125 solvent module, model 508 autosampler and a model 168 detector. The detector was set to monitor absorbance over the range 270–420 nm. Reverse-phase chromatography was performed with a 1.5 cm guard column (Applied Biosystems, Foster City, CA, USA), and a Wakosil II 5 C18RS 5 μ 4.0 × 250 mm analytical column (SGE Analytical Science Pty Ltd, Victoria, Australia) equilibrated with methanol/(2% aqueous acetic acid), 7 + 3 (v/v). A total of 20 μl of sample solution was injected and separated under isocratic conditions at a flow rate of 0.5 ml min^{-1}. Normal-phase chromatography was performed using a 1.5 cm silica guard column (Applied Biosystems), and a Zorbax silica 4.6 × 150 mm analytical column with isocratic 9 + 1 chloroform/(1% acetic acid in methanol) (v/v) at a flow rate of 0.5 ml min^{-1}.

HPLC-MS experiments were conducted with the same analytical columns and mobile phase conditions as described above, except with atmospheric pressure chemical ionization (APCI) and mass spectrometric detection. The instrumentation consisted of a model P4000 pump, AS3000 autosampler with a 100 μl sample loop (Thermo Separations, San Jose, CA, USA) and a model LCQ quadrupole ion-trap mass spectrometer with APCI interface (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The APCI source conditions were as follows: vaporizer temperature, 350°C; sheath gas flow rate, 70; discharge current 5.00 μA; and capillary temperature, 170°C.

The fusarin standards were examined after exposure to conditions normally encountered during ELISA. This was done in order to observe changes in the fusarin preparations under such conditions. Standard fusarin A or C were diluted in phosphate buffered saline (PBS: 0.01 M sodium phosphate, 0.145 M sodium chloride, pH 7) to a concentration of 10 μg ml^{-1} under ambient light, then aliquots were placed in a polystyrene microplate. The microplate was covered with a transparent film and stored in the dark at ambient temperature. At various time points up to 4 h the plate was removed from the dark and aliquots were taken and tested by reverse-phase HPLC-PDA. This effectively mimicked the ELISA situation, where incubations were conducted in the dark but manipulations were done under ambient light.

Preparation of fusarin C protein conjugates

Protein conjugates of fusarin C were synthesized by linking the hydroxyl groups of the toxin to the primary amines of the proteins using a carbodiimide technique. The immunogen was a conjugate of fusarin C with OVA (FU-OVA). Where possible manipulations were done, and samples stored, in the dark. On the day of the reaction 10 mg of fusarin C in methylene chloride were concentrated to the consistency of a glass under reduced pressure. Acetone, 0.16 ml, was added to solubilize the fusarin and 110 mg of 1,1′-carbonyldiimidazole was added. The reaction was sealed and held at ambient temperature for 1 h, after which 0.04 ml of water were added, followed by 0.8 ml of OVA solution (18.8 mg ml^{-1} in 0.1 M sodium bicarbonate buffer, pH 8.6). The mixture was incubated for 24 h at 4°C, then dialyzed against three sequential changes of 0.1 M PBS to remove unbound fusarin. The protein concentration of the conjugates were determined by

assay with bicinchoninic acid (Pierce, Rockford, IL, USA). The FU-OVA was diluted to 2 mg ml^{-1} with 0.1 M PBS, then freeze-dried and sent to Harlan Bioproducts for Science (Madison, WI, USA) for administration into mice. The test antigen, a conjugate of fusarin C with BSA (FU-BSA) was prepared in a similar fashion.

Immunizations and screening for fusarin-specific antibodies by CI-ELISA

All animal work and cell culture experiments were conducted at Harlan Bioproducts for Science (Madison, WI, USA). Female Balb/C mice were initially immunized by injection of 100 μg FU-OVA per animal using the same procedures as described previously for production of deoxynivalenol (DON) antibodies using DON-OVA conjugates (Maragos and McCormick 2000).

A CI-ELISA was developed for screening of mouse sera and culture supernatants for the presence of antibodies. For screening assays 0.1 ml of FU-BSA conjugate, 1 μg ml^{-1} in 0.05 M sodium phosphate buffer (pH 7), was added to wells of high binding polystyrene microtitre plates (Immulux HB flat bottom microplates, Dynex Technologies, Chantilly, VA, USA) and allowed to attach overnight at 4°C. In later assays, for comparison of different clones and for cross-reactivity studies, the level of FU-BSA coated was further reduced to 100 ng ml^{-1}. After washing the coated plate twice with 0.32 ml Tween-PBS (0.02% Tween-20 in 0.01 M PBS pH 7), 0.32 ml of PVA-PBS (1% polyvinyl alcohol in 0.01 M PBS) were added and allowed to incubate at ambient temperature for 2 h in the dark. During this incubation, test solutions were prepared. The test solutions consisted of 0.06 ml of toxin standard solutions (or PBS control) mixed with 0.06 ml of serum (or culture fluid) diluted in OVA-PBS (1% w/v OVA in 0.01 M PBS) in the wells of a non-binding polypropylene microwell plate (Corning, Inc., Corning, NY, USA). The wells of the polystyrene (FU-BSA-coated) plate were washed twice with Tween-PBS and 0.1 ml of test solution was transferred into each well. After incubation at ambient temperature for 30 min in the dark the plate was washed three times and 0.1 ml of goat anti-mouse peroxidase conjugate (diluted 1:2000 in OVA-PBS) was added. The plate was incubated for 30 min at ambient temperature in the dark then washed four times before addition of 0.1 ml of the substrate, *o*-phenylenediamine (OPD). The OPD solution was prepared by combining 0.02 ml of 30% H_2O_2 and 20 mg OPD in 50 ml of citrate-phosphate buffer (0.05 M citrate, 0.1 M phosphate, pH 5.0). After 5 min at ambient temperature the reaction was stopped by the addition of 0.1 ml of 1 N hydrochloric acid. Colour development was determined by measuring the absorbance at 490 nm using a Synergy HT microplate reader (Bio-Tek, Winooski, VT, USA).

Production and purification of monoclonal antibodies

Mice having sera with antibodies reactive to fusarin C were sacrificed and aseptically splenectomized. Spleenocytes were chemically fused with Balb/C non-immunoglobulin secreting (NS-1) myeloma cells using polyethylene glycol. Fused cells were plated in HAT selection media. After 11 days, HAT resistant cultures were isolated and screened for anti-fusarin activity by CI-ELISA. Positive cultures were subsequently cloned, expanded and frozen. Seven clones that screened positive were designated 1–5, 1–7, 1–21, 1–25, 1–30, 1–38, and 1–43 and were expanded for ascites fluid production using established procedures (Hoogenraad et al. 1983). The ascites fluid was partially purified by ammonium sulfate precipitation using procedures described previously. Briefly, the ascites fluid was delipidated with a mixture of dextran sulfate and calcium chloride then twice-precipitated with ammonium sulfate, followed by dialysis against 0.1 M PBS before freeze-drying and storing at −20°C (Maragos and McCormick 2000).

Results and discussion

Stability of fusarins A and C in buffer

Because of the reported rearrangement of fusarins and instability to light we endeavoured to determine the relative stability of fusarins A and C that might be expected under ELISA conditions. This was attempted in order to determine whether the fusarins might survive in aqueous solution long enough to be measured by an immunochemical method. For these experiments either fusarin A or C was held in PBS in the wells of a microtitre plate for up to 4 h. Samples were periodically withdrawn from the plate and assayed by reverse-phase HPLC with photodiode array (PDA) detection. Between collection of samples the plate was kept in the dark, but sampling was done under ambient light. As described in the Introduction, the fusarins have been reported to rearrange rapidly in solution, giving rise to multiple chromatographic peaks having the absorption band at about 360 nm. While the bulk of the literature on fusarins deals with normal-phase chromatography, generally with chloroform/methanol mobile phases, we chose to base the examination upon reverse-phase chromatography. This was done so as to ensure that the mobile phase would be miscible with

the aqueous ELISA buffers used in the stability testing.

With normal-phase chromatography, multiple peaks were observed for fusarin C that changed in size over time (Jackson et al. 1990). We observed a similar situation with reverse-phase chromatography. Figure 2 shows reverse-phase HPLC-PDA chromatograms of fusarin A and C solutions after having been held for 2 h in PBS. The spectra of several of the peaks were consistent with those of authentic fusarin C and of fusarin X, which have similar spectra (Scott et al. 1986; Lu and Jeffrey 1993). This is consistent with an interpretation that the chromophore may have rearranged but was not substantially destroyed.

The fusarin A and C standards were also evaluated by HPLC-MS. The major chromatographic peaks for fusarin A at 15.3, 18.5, 22.9, and 25.2 min all contained the mass/charge (m/z) ratio corresponding to protonated fusarin A ($m/z = 416$). The major chromatographic peaks for fusarin C at 13.7, 15.5, and 16.8 min contained the $m/z = 432$ consistent with the presence of protonated fusarin C. The HPLC-MS and HPLC-PDA data are consistent with the interpretation that our fusarin A starting material was a mixture of several isomers having the mass of fusarin A and UV spectrum similar to fusarin C. To our knowledge the UV light spectrum of fusarin A has not been published, although the absorption maximum and extinction coefficient at 352 nm have been reported (Steyn and Vleggaar 1985). The UV spectra we observed for fusarin A were similar to those reported for fusarin C, which might be expected as both contain the pentaene chromophore. The fusarin C starting material was a mixture of several isomers having the same mass and UV spectra as fusarin C, as well as containing 13.3% of what may have been fusarin A as an impurity (with fusarin A possibly being responsible for the peaks at 18.5, 22.9, and 25.2 min in the fusarin C).

When the fusarin A and C preparations were placed in buffer and monitored by HPLC-PDA they behaved quite differently. Figure 3 shows the behaviour of the four major fusarin peaks for either fusarin A or fusarin C over time in PBS. For fusarin

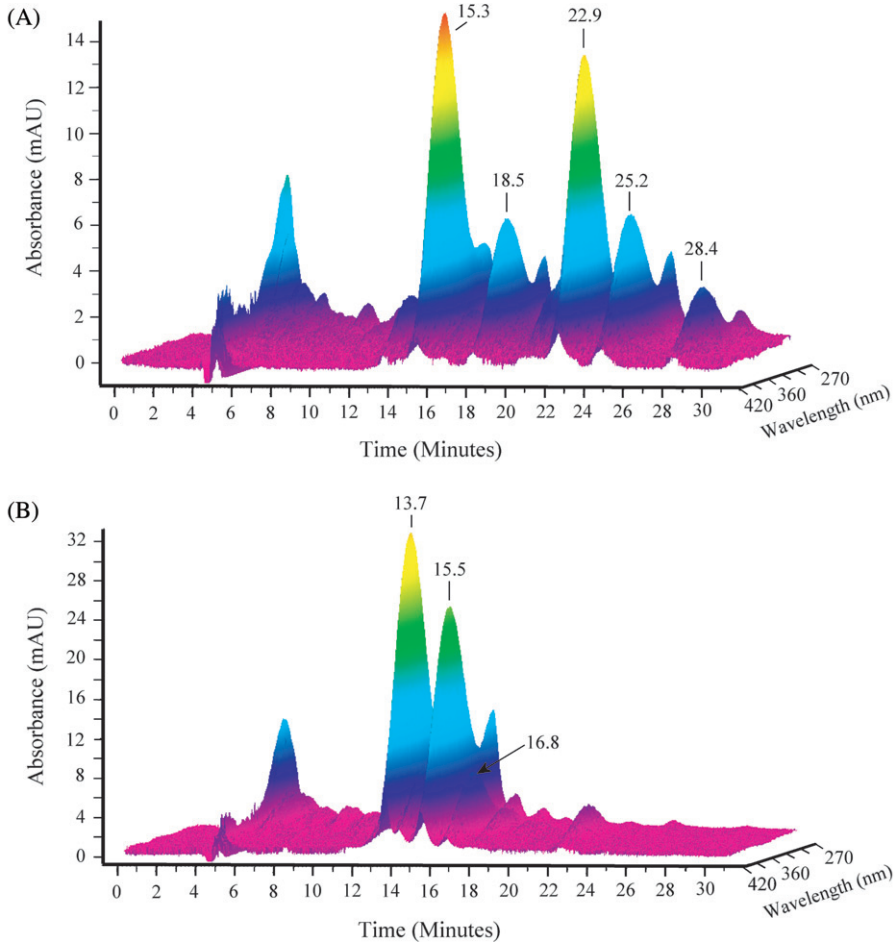


Figure 2. Reverse phase HPLC-PDA chromatograms of fusarins A (A) and C (B), after being held 2 h in PBS buffer. Retention times of the major fusarin peaks are indicated.

A there was a rapid decline in the peak with a retention time of 22.9 min and a concomitant increase in the peak with a retention time of 15.3 min. A similar phenomenon was also observed for two other peaks, with retention times of 25.2 and 18.5 min (data not shown). For fusarin C no such phenomena were observed: the major peaks did not change in relative proportion within the 4 h of the experiment (Figure 3B).

The data show that the chromophore present in the fusarin A preparation rapidly redistributed amongst the existing chromatographic peaks, while the fusarin C preparation did not. The fusarin A preparation appeared to reach an equilibrium within 1 h. We speculate that, unlike the fusarin A, the fusarin C mixture may have attained an equilibrium among the various isomers before the first measured time point (2 min after mixing the toxin with buffer), which may explain why we did not observe further changes. As indicated previously fusarin F has been suggested to rearrange to fusarin C (Savard and Miller 1992). The lack of a substantial change in the peaks observed with our fusarin C preparation over time suggests that, if fusarin F were present and if it were to rearrange to fusarin C, the process had already reached equilibrium before the first time point was collected.

The data in Figure 3 also indicate that although there was redistribution of the location of the chromophore, overall the chromophore was relatively stable over the 4-h span under these conditions. It is important to note that in these experiments the fusarins were handled so as to mimic the ELISA conditions. Specifically, sampling from the plates was done under ambient light, and the plates were kept in the dark between sample collections. All experiments were at ambient temperature. The results suggest that the fusarin preparations, especially fusarin A, may have undergone an equilibration process. The products presumably included the various stereo-isomers resulting from rearrangement of the double bonds in the chromophore, however these do not preclude other rearrangements to the basic fusarin structure, such as skeletal rearrangements from backside electrophilic attack by an hydroxyl group on the epoxide substituent of the pyrrolidone ring system, leading to rearrangements between isomers of fusarins C, D and F. Regardless of the identity of the products, which we do not know and which are likely to be complex mixtures, the preparations

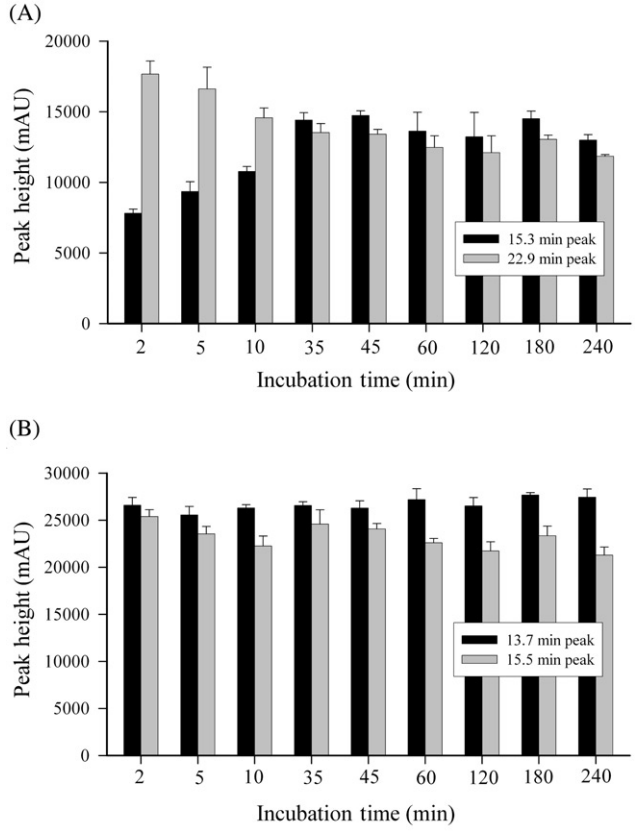


Figure 3. Stability of fusarin preparations over 4 h in buffer. Fusarin standards were incubated for various times in PBS buffer before injection onto reverse-phase HPLC. Data on the vertical axis represent peak heights of the major fusarin peaks with the indicated retention times as shown in Figure 2. Error bars represent standard deviations from triplicate experiments. (A) fusarin A, (B) fusarin C.

were sufficiently stable that most of the 'fusarin' (those compounds having the *m/z* and UV spectra of authentic fusarins) would have been expected to survive under ELISA conditions.

Preparation of fusarin C protein conjugates

Ovalbumin (OVA) and BSA conjugates of fusarin C were synthesized by linking the hydroxyl groups of the toxin to the primary amines of the proteins using a carbodiimide technique. The OVA conjugate (FU-OVA) was administered to mice to generate an immune response, while the BSA conjugate (FU-BSA) was used as the coating antigen for the screening of fusarin antibodies. Of the ten mice that were initially immunized five showed titres indicating a serum response to the FU-OVA. Of these mice four had a response that could be influenced by the presence of fusarin C added to the incubation mixture in a competitive assay. The mouse with the most sensitive response to fusarin C was selected for splenectomy and hybridoma production. From this mouse three hybridomas were isolated, one of which showed good binding to the FU-BSA and which could be displaced from it in competitive assays with fusarin C. Unfortunately, that hybridoma could not be isolated during the cloning procedure.

Rather than proceed with hybridoma production with one of the three remaining mice, which did not have strong responses to free fusarin C, the process was repeated in a second trial. The second trial involved 20 mice, 19 of which developed antibodies that bound the FU-BSA test antigen. Of these mice 11 had a response that could be influenced by the presence of fusarin C added to the incubation mixture in a competitive assay. The mouse with the best response to fusarin C was selected for splenectomy and hybridoma production. From this mouse 45 hybridomas were isolated, 16 of which showed good binding to the FU-BSA and which could be displaced from it in competitive assays with fusarin C. From this number, seven fusarin antibody-producing cell lines were isolated and used to produce larger amounts of antibody in mouse ascites.

Response of Mabs to fusarins A and C

The seven monoclonal antibodies (Mab) that were produced were tested for response to fusarins A and C in a competitive indirect (CI) ELISA format. The ELISAs for three of the antibodies, those from cell lines 1–38, 1–30, and 1–5 showed sensitive responses to fusarin C, with IC_{50}'s ranging from 1 to 3.6 ng ml^{-1} (Table I). Although the immunogen was a fusarin C-OVA conjugate, all of the antibodies cross-reacted with fusarin A (Table I). The cross reactivities for fusarin A relative to fusarin C (100%) were: 44.8, 51.4, 41.1, 174.0, 62.6, 78.2, and 98.0%, for Mabs 1–38, 1–30, 1–5, 1–7, 1–43, 1–25, and 1–21, respectively. In general the antibodies with the greatest sensitivity for fusarin C (i.e. 1–38, 1–30, and 1–5) showed lower cross-reactivity to fusarin A. Interestingly, one of the Mabs (1–7) showed a greater reactivity for fusarin A than for fusarin C. The relative responses of the two most sensitive fusarin C antibodies (1–38 and 1–30) and Mab 1–7 are shown in Figure 4. The limits of detection (LOD) of the assays for fusarin C in buffer, defined as the concentrations that were three standard deviations from the responses of the toxin-free controls, were 0.24, 0.31 and 1.03 ng ml^{-1} for Mabs 1–38, 1–30, and 1–5, respectively. For fusarin A the calculated LODs in buffer were 0.43, 0.43, and 1.82 ng ml^{-1} for Mabs 1–38, 1–30, and 1–5, respectively.

Table I. Response of seven monoclonal antibodies to fusarins A and C.

Antibody	Fusarin	$IC_{50} \pm SD$ (ng ml^{-1})[a]
1–5	A	8.7 ± 0.7
	C	3.6 ± 0.4
1–7	A	13.4 ± 2.5
	C	23.4 ± 4.5
1–21	A	68.0 ± 10.9
	C	66.7 ± 10.3
1–25	A	40.1 ± 4.8
	C	31.4 ± 5.7
1–30	A	3.9 ± 0.3
	C	2.0 ± 0.1
1–38	A	2.3 ± 0.1
	C	1.0 ± 0.1
1–43	A	46.1 ± 9.8
	C	28.9 ± 3.5

[a]Concentration of toxin required to inhibit colour development by 50% (IC_{50}) and the associated standard deviations ($n = 4$ plates for all antibodies except 1–38, where $n = 8$ plates).

In conclusion, we have reported the development of seven Mabs for detecting fusarins A and C. While the various fusarin isomers appeared to undergo rearrangements that affected the HPLC chromatography of these toxins, overall the chromophore was generally stable under the conditions used here for immunoassay. The conjugated fusarin C was also sufficiently stable to allow us to obtain an immune response capable of recognizing this toxin and/or its isomers in solution. Three of the Mabs in particular were sensitive for fusarin C, with IC_{50}'s ranging from 1.0 to 3.6 ng ml^{-1}. All seven of the Mabs cross-reacted with fusarin A to various degrees suggesting the antibodies have some latitude in the structures that they will accept. Because extensive surveys of the prevalence of fusarins in foods have not been reported, we do not yet have a clear indication of what levels might be expected to be encountered during a survey. However, given the low limits of detection reported for several of the Mabs described

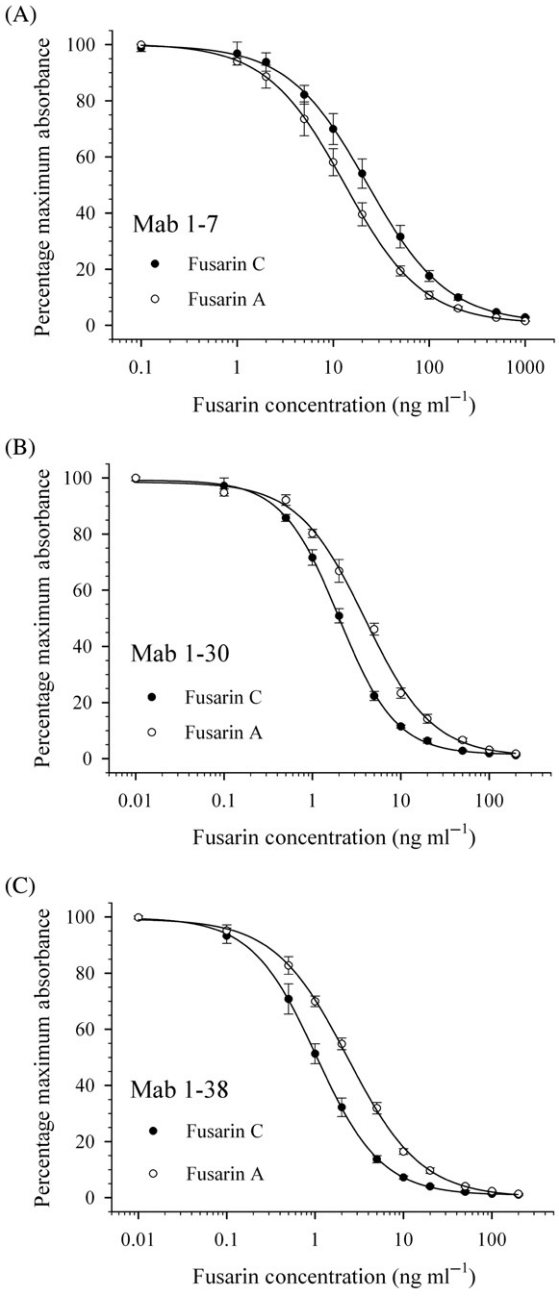


Figure 4. Response of three monoclonal antibodies (Mab) to fusarins. (A) Mab 1–7, (B) Mab 1–30, (C) Mab 1–38. Each curve represents the fit of a four parameter logistic dose-response curve to the data from four to eight replicate plates. Error bars represent ±1 standard deviation from the mean. The IC_{50}'s corresponding to these curves are included in Table I.

here, we expect that one or more of them will be useful in the further development of screening assays for fusarins in cereal grains and foods.

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